Cross-Linking of Maize Walls by Ferulate Dimerization and Incorporation into Lignin

John H. Grabber,* John Ralph, and Ronald D. Hatfield

U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1925 Linden Drive West, Madison, Wisconsin 53706

Cross-linking of xylans and lignin by ferulates was investigated with primary maize walls acylated with 2% ferulate and with ferulate ethyl esters. Peroxidase-mediated coupling of wall ferulate and ethyl ferulate yielded mostly 8-coupled products, including three new dehydrodimers. Significant quantities of 5−5-coupled diferulate formed only within walls, suggesting that matrix effects influence dimer formation. Over 60% of wall ferulate dimerized upon H_2O_2 addition, suggesting that xylan feruloylation is highly regulated during wall biosynthesis to permit extensive dimer formation at the onset of lignification. During lignification, ferulate and 5−5-coupled diferulate copolymerized more rapidly and formed fewer ether-linked structures with coniferyl alcohol than 8−5-, 8−*O*-4-, and 8−8-coupled diferulates. The potential incorporation of most ferulates and diferulates into lignin exceeded 90%. As a result, xylans become extensively cross-linked by ferulate dimerization and incorporation to lignin, but only a small and variable proportion of these cross-links is measurable by solvolysis of lignified walls.

Keywords: *Gramineae; Zea mays; ferulic acid; diferulic acids; cell wall; lignin; dehydrogenation polymer; cross-linking*

INTRODUCTION

Ferulic acid is ester linked to the C5 hydroxyl of α-L-arabinosyl side chains on grass arabinoxylans. Xylans become cross-linked by peroxidase/H_2O_2-mediated radical coupling of ferulate (FA) into 8−8-, 8−5-, 8−*O*-4-, and 5−5-coupled dehydrodimers (Ishii, 1991; Ralph et al., 1994; Grabber et al., 1995). Biomimetic lignification systems (Grabber et al., 1995; Ralph et al., 1992b; Quideau and Ralph, 1997) and FA−lignin isolates from grasses (Ralph et al., 1995; Jacquet et al., 1995) have revealed that ferulate and diferulate (DFA) esters copolymerize with monolignols, thereby cross-linking xylans to lignin. These cross-links probably contribute to wall stiffening and growth cessation in plants and to poor degradation of cell walls by hydrolytic enzymes (Tan et al., 1992; Schopfer, 1996; Musel et al., 1997; Grabber et al., 1998a,b). FA cross-links also alter the physical properties of foods (Oosterveld et al., 1997; Schooneveld-Bergmans et al., 1999).

The fate of FA and DFA is difficult to track because FA deposition, dehydrodimerization,and copolymerization into lignin are probably overlapping processes during cell wall formation in grasses. This difficulty is further compounded by our inability to fully recover or characterize, by solvolytic or spectroscopic methods, FA in lignified walls. Each step of this process, however, can be isolated and elucidated using cell suspensions as a model system for cell wall development in grasses. In a previous study (Grabber et al., 1995), feruloylated primary walls and synthetically lignified walls prepared from maize cell suspensions provided cursory information about the potential extent of DFA− and FA−lignin cross-linking in grass cell walls. This paper describes subsequent studies conducted with this cell wall model system and ethyl esters of FA and DFA, which allowed us to further delineate FA dehydrodimerization in cell walls, the kinetics of FA and DFA incorporation into lignin, and the proportions of alkali-labile FA− and DEA−lignin cross-products formed in walls.

* Author to whom correspondence should be addressed (e-mail jgrabber@facstaff.wisc.edu).

MATERIALS AND METHODS

In Situ Studies with Feruloylated Maize Walls. Cells from 350 mL maize cell suspensions (*Zea mays* cv. Black Mexican), collected at the early stationary growth phase, were suspended in ice-cold piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.8, 10 mM) and ruptured by two passages through a Parr nitrogen bomb maintained at 1500 psi (Grabber et al., 1995). Wall fragments were collected on a nylon mesh (20 μm) and washed successively with buffer and water to remove cytoplasmic material. Walls [∼6.4 g of dry weight (dw), containing peroxidases and ∼125 mg of ferulate] were suspended in 500 mL of 25 mM homopiperazine-*N*,*N*′-bis-2-(ethanesulfonic acid) (HOMOPIPES) buffer (pH 5.5) with 5 μkat of glucose oxidase (Sigma, G9010). Glucose (400 mg) in 50 mL of HOMOPIPES buffer was added to the stirred wall suspension over a 2 h period to generate (via glucose oxidase) ∼3.5 equiv of H_2O_2/mol of FA. One hour following glucose additions, walls were collected on a nylon mesh, washed with water, and stirred overnight at 4 °C in 2 volumes of 200 mM $CaCl_2$. The following morning, walls were collected on a nylon mesh and washed with 200 mM $CaCl_2$ followed by water to remove loosely bound peroxidases. After draining, walls (560 mg of dw, containing ∼11 mg of FA) were suspended in 50 mL of HOMOPIPES buffer with 2 μkat of glucose oxidase and lignified with coniferyl alcohol **13** (5 to 80 mg) utilizing wall-bound peroxidases and H_2O_2 generated by the glucose/glucose oxidase system. Coniferyl alcohol (0.83 mg/mL) and glucose (0.83 mg/mL) were dissolved in HOMOPIPES/acetone (6:1) buffer and added at a rate of 12 mL h^{-1} to the stirred wall suspensions. Nonlignifed controls were incubated in HOMOPIPES buffer. After stirring for a total of 24 h, walls were collected on glass-fiber filters (1.2 μm porosity) and washed

10.1021/jf0006978
Published on Web 11/28/2000

with water followed by acetone and air-dried. Cell wall lignification experiments were conducted two times.

Nonlignified and lignified walls (50−75 mg) were hydrolyzed for 20 h with 2 N NaOH (4 mL) at room temperature to release ester-linked ferulates. Selected samples (50 mg) were also hydrolyzed at 170 °C for 2 h in Teflon vials with 4 N NaOH (4 mL, with 0.5% anthraquinone) to release ester- and ether-linked FA. 2-Hydroxycinnamic acid (0.1 mg) was added as an internal standard. Hydrolysates were acidified to pH <2 with 12 M HCl and extracted with diethyl ether (4 mL, two times). Dried extracts were derivatized for 30 min with pyridine (5 μL) and BSTFA (25 μL) at 60 °C and injected (1−2 μL) into a GC-FID (Perkin-Elmer 8500, split ratio 1:50). Compounds were separated using a 0.25 mm × 30 m (0.25 μm film thickness) DB-1 column (J&W Scientific) or a 0.2 mm × 30 m (0.2 μm film thickness) SPB-5 column (Supelco) using He as the carrier gas (0.8 mL min^{-1}). The columns were held at 200 °C for 1 min, ramped at 5 °C min^{-1} for 8 min to elute hydroxycinnamate monomers, then ramped at 30 °C min^{-1} to 310 °C, and held for 12 min to elute DFA. The injector and FID were set at 310 °C. FA and DFA concentrations were calculated using previously determined GC-FID response factors (Ralph et al., 1994). Response factors for (*Z*) and (*E*,*Z*) isomers (**2**, **9**, and **11**) were assumed to be identical to those of their corresponding (*E*) and (*E*,*E*) isomers (**1**, **7**, and **8**). The response factor of DFA **10** was assumed to be similar to that of DFA **3**. Similar hydrolysis, derivatization, and GC conditions were used to obtain EI-MS data with an HP 5970 mass-selective detector and a Polaris GCQ Plus ion-trap detector.

The quantity of an FA or DFA incorporated into lignin was estimated as the difference in acids recovered from nonlignified and lignified walls following room temperature alkaline hydrolysis. Concentrations of acids recovered from room temperature hydrolysis were used without adjustment because 2-hydroxycinnamate (the internal standard), FA, and DFA undergo similar losses through the procedure. Typically, the quantities of ether-linked FA and DFA are estimated as the difference in acids recovered from lignified walls following room temperature and high-temperature alkaline hydrolysis. These acids, however, undergo differential loss during high-temperature hydrolysis so some means of adjusting their concentrations is needed to obtain accurate estimates of ether-linked FA and DFA. In an earlier study (Grabber et al., 1995), we observed that FA and DFA are quantitatively (>97%) released from nonlignified walls by room temperature hydrolysis. Therefore, the ratios of acids recovered from nonlignified walls following room temperature and high-temperature hydrolysis were used to correct for losses of FA and DFA during high-temperature hydrolysis of lignified walls. The quantity of ether-linked acids in lignified walls was then calculated by subtracting the concentration of acids recovered by room temperature hydrolysis from the adjusted concentrations of acids recovered from high-temperature hydrolysis. All FA and DFA values for lignified walls were also adjusted to account for increases in wall mass caused by lignification.

In Vitro Studies with Monolignols and Ethyl Esters of FA and DFA. Crude wall-bound peroxidases were extracted by mixing cell walls from maize cell suspensions with 3 M aqueous LiCl to form a thick suspension. After mixing overnight at 2 °C, a crude peroxidase extract was recovered by filtration (Miracloth), dialyzed overnight at 2 °C in acetate buffer (50 mM, pH 5.5), and concentrated on ice by ultrafiltration (10 kDa, YM10 Amicon). The peroxidase extract was then loaded onto an HW-650 DEAE column (2.5 cm × 20 cm) and eluted with a 0−1 M NaCl gradient in 50 mM Tris buffer (pH 7.0). Fractions containing peroxidase activity were pooled and concentrated by ultrafiltration (10kDa, YM10 Amicon). The purified peroxidases (3 μg) and H_2O_2 (10 μmol) were stirred in 10 mL of HOMOPIPES buffer (10 mM, pH 5.5) for 10 min to partially dimerize a mixture of (*E*)-ethyl FA **1c** (4 μmol) and (*Z*)-ethyl FA **2c** (1 μmol). The purified peroxidases (6 μg) and H_2O_2 (10 μmol in 50 μL) were also stirred in 10 mL of HOMOPIPES buffer (10 mM, pH 5.5) for 15 min to partially copolymerize mixtures of coniferyl alcohol (5 μmol) with FA **1c**, 8−5-coupled DFA **7c**, or 5−5-coupled DFA **8c**. Both in vitro experiments were done twice. Reactions were stopped by adding 0.25 mL of 12 M NaOH and incubated for 20 h under N_2 to hydrolyze ethyl ester groups. After hydrolysis, samples were acidified (pH <2) with 12 M HCl and extracted with 5 mL of diethyl ether (two times). Dried extracts were silylated and subjected to GC-FID analysis as described previously.

RESULTS AND DISCUSSION

Saponification of nonlignified primary walls from maize cell suspensions typically releases 18−22 mg g^{-1} FA monomers and dimers, comprising 65% of (*E*)-FA (**1a**), 20% of (*Z*)-FA (**2a**), and 15% of various diferulates (Grabber et al., 1995, 1998a). DFA are probably formed by wall-bound peroxidases, although some dimerization may occur within golgi vesicles just prior to xylan deposition into walls (Myton and Fry, 1995). Once deposited within walls of maize cell suspensions and other grass tissues, FA undergo extensive dimerization if suitable peroxidases and H_2O_2 are present (Grabber et al., 1995; Schopfer, 1996; Gonzalez et al., 1999).

Peroxidase-Mediated Dimerization of FA. The concentrations of both (*E*)- and (*Z*)-FA in walls were reduced as the activity of wall-bound peroxidases was stimulated by the gradual generation of H_2O_2 by glucose oxidase. The main coupling products recovered after saponification were, however, DFA **3a**−**8a**, typically derived from homocoupling of (*E*)-FA (**1b**) (Figures 1 and 2). These DFA were described previously (Ralph et al., 1994; Markwalder and Neukom, 1976; Hartley and Jones, 1976), and their mass spectra are shown in Figure 3. As noted previously (Ralph et al., 1994), 8−5-coupling of (*E*)-FA forms a benzofuran (**7b**) in walls, but saponification and silylation give a mixture of **5a** and **7a** in varying proportions and a decarboxylated product (**12a**). The 8−8-coupled DFA **3a** (aryltetralin) and **4a** are recovered in varying but reproducible proportions from a variety of grasses following saponification and silylation (Ralph et al., 1994; J. H. Grabber, unpublished results), suggesting that these dimers exist as their corresponding ester analogues **3b** and **4b** in cell walls. The 8−*O*-4- and 5−5-coupled DFA **6b** and **8b** also remain intact during saponification and derivatization.

Small quantities of (*Z*)-8−5-coupled diferulic acid (**9a**) and (*E*,*Z*)-5−5-coupled diferulic acid (**11a**) were identified for the first time by their mass spectral fragmentation patterns and by comparing their GC retention times to those of (*Z*)-isomers formed by UV exposure of DFA **7a** and **8a**. These isomers comprised ∼20% of the 8−5- and 5−5-coupled DFA recovered from cell walls. In contrast, 8−8- or 8−*O*-4-coupled DFA with (*Z*) side chains were not detected. A new 8−8-coupled tetrahydrofuran structure, **10a**, was also tentatively identified by its mass spectral fragmentation pattern. This dimer was probably formed by 8−8-coupling of at least one (*Z*)-FA; in peroxidase/H_2O_2 oxidations of ethyl (*Z*)- and (*E*)-FA (**1c** and **2c**), the proportion of **10a** increased in concert with the proportion of (*Z*)-FA used in the reaction mixture. The remaining minor peaks had mass spectra charactistic of truxillic acids (Jacquet et al., 1995; Hartley et al., 1990).

DFA with (*Z*) side chain configurations are scarce because (*Z*)-FA typically comprise <20% of the monomers in grass walls (Akin et al., 1992; Turner et al., 1993). In addition, phenylpropanoid coupling reactions favor the formation of dimers with (*E*) rather than (*Z*) side chain configurations. For example, in non-8−8-hetero-couplings of coniferyl alcohol, the (*Z*)-isomer

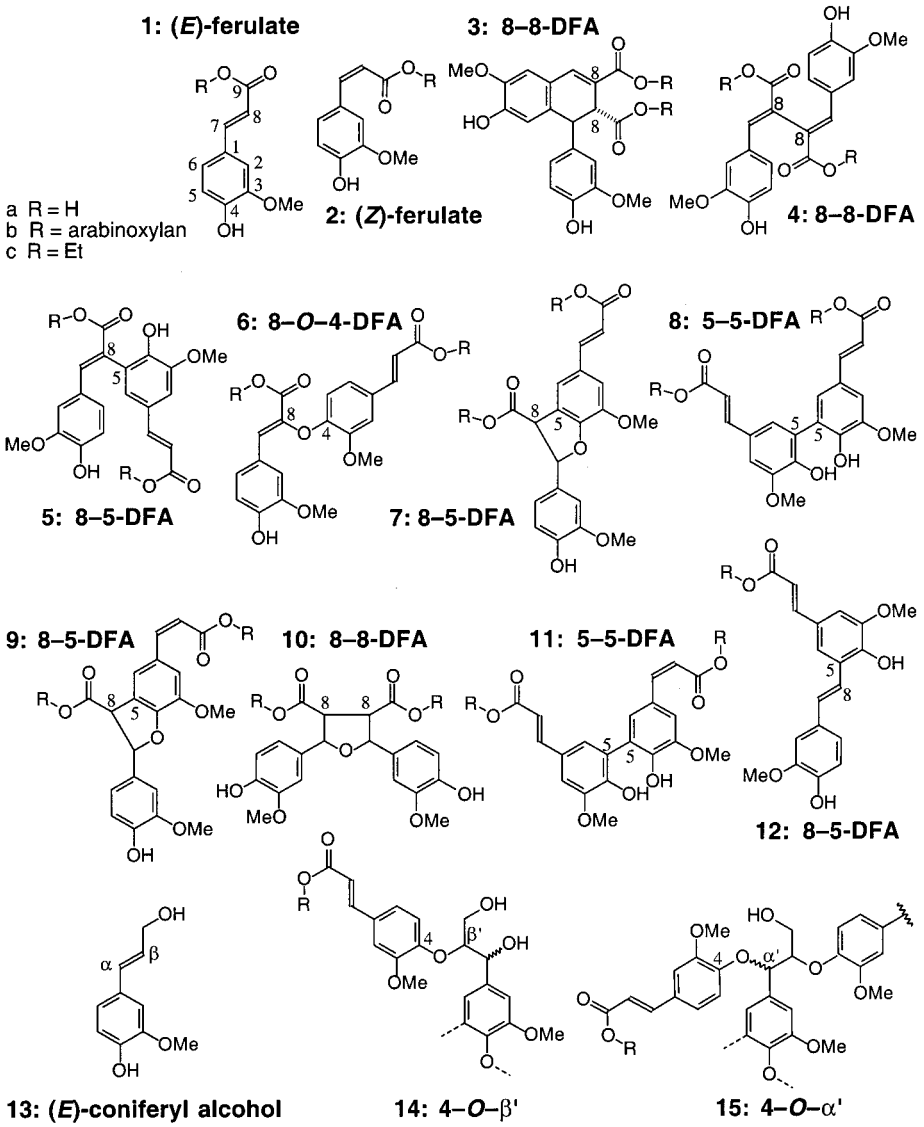

Figure 1. Ferulates (FA, **1** and **2**) and diferulates (DFA, **3**−**12**) detected in primary walls isolated from maize cell suspensions. During lignification of cell walls with coniferyl alcohol (**13**), some FA (and DFA) become attached to lignin by 4-O-β' (**14**) and 4-O-α' (**15**) linkages.

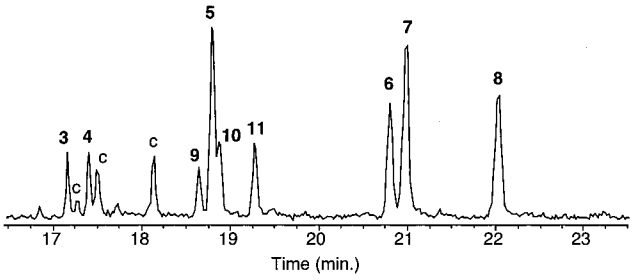

Figure 2. GC-MS total ion chromatogram of DFA (TMS-derivatized) released from primary maize walls by room temperature alkaline hydrolysis. Peaks c are assigned, without further authentication, to cyclodimers on the basis of their mass spectra.

usually couples at the 8-position, allocating the (*E*)-isomer the less favored 5- and 4−*O*-coupling positions (Ralph and Zhang, 1998). By extrapolation, in 8−5-heterocouplings of FA, the (*Z*)-isomer would probably couple at its 8-position. The stereochemistry of the (*Z*)-FA moiety would be then lost; intramolecular phenolate attack on the quinone methide intermediate then forms the benzofuran ring in the *trans* configuration (Ralph et al., 1994). The resulting dimer **7b** is identical to that formed by 8−5-homocoupling of (*E*)-FA. In the case of 8−*O*-4-heterocouplings, the more thermochemically stable (*E*) configuration forms via the quinone methide intermediate. The product, (*Z*,*E*)-8−*O*-4-coupled diferulate (**6b**), is identical to that formed by homocoupling of (*E*)-FA. [The (*Z*,*E*) designation is required by rules of stereo nomenclature (Ralph et al., 1994).] Similar mechanisms are involved in the formation of 8−8-coupled DFA from homo- and heterocouplings of (*Z*)- and (*E*)-FA.

FA trimers or oligomers were not detected in saponification extracts by chemical ionization mass spectrometry or by fast atom bombardment mass spectrometry. Apparently, oxidative coupling reactions of cell wall FA, in the absence of monolignols, are limited to dimerization.

As observed in our previous study (Grabber et al., 1995), FA moieties of dehydrodiferulates formed in H_2O_2-treated walls were linked primarily by 8−5-couplings (52%), with 5−5-, 8−*O*-4-, and 8−8-couplings each contributing ∼16% of the total (Table 1). In contrast, oxidation of ethyl FA (**1c** and **2c**) with maize peroxidase and H_2O_2 produced more 8−5-coupled DFA (77%), comparable levels of 8−8- and 8−*O*-4-coupled

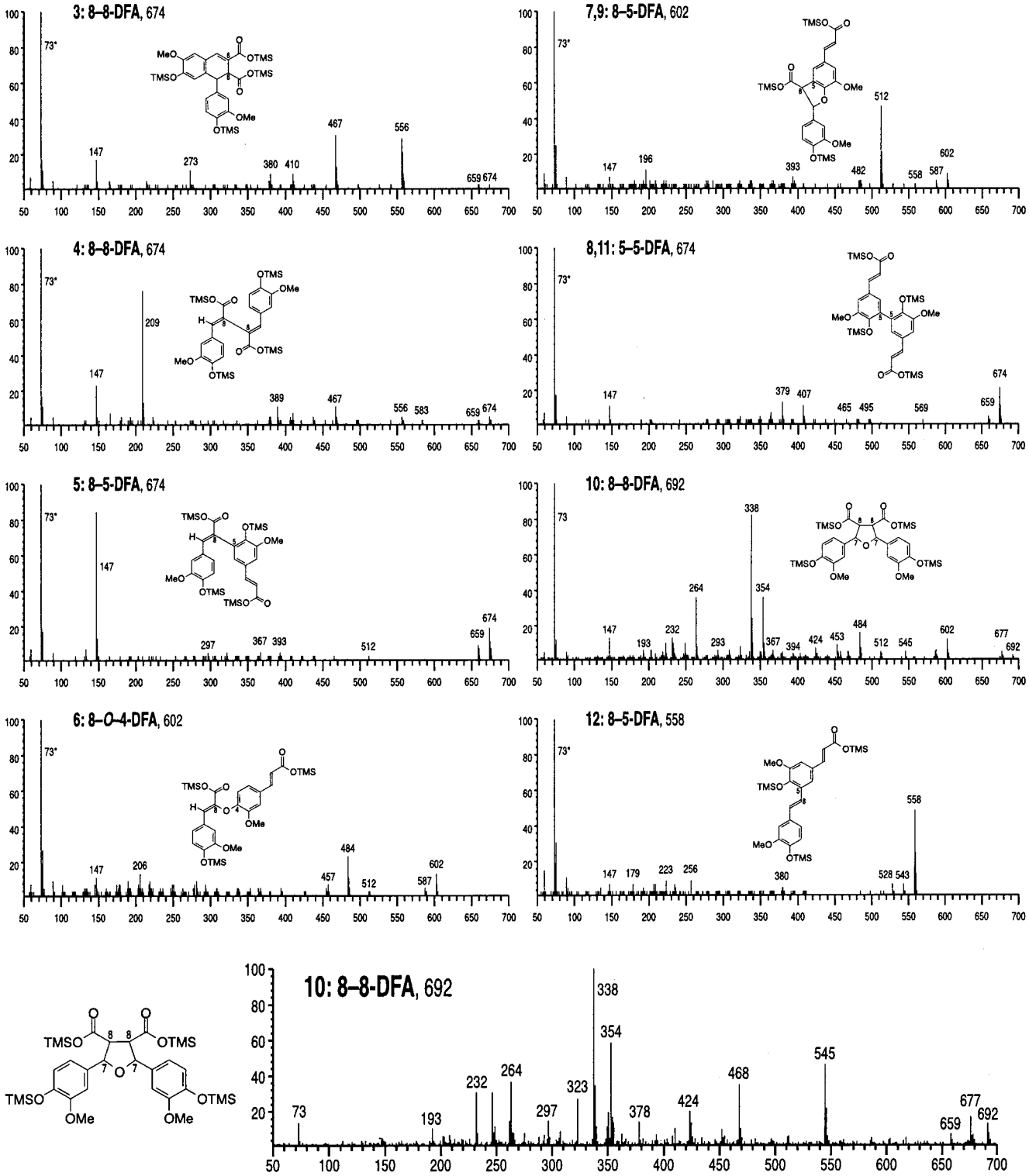

Figure 3. Electron ionization mass spectra of DFA collected with a mass selective detector (dimers **3**−**12**) and an ion-trap detector (dimer **10**). The intensity of the TMS ion (i.e., 73*) for all spectra except **10** was reduced by half to enhance the visibility of other ions.

DFA (16 and 6%, respectively), and extremely low levels of 5−5-coupled DFA (0.3%). In these experiments, ethyl FA was rapidly oxidized for a brief period to favor the formation and recovery of DFA over larger polymerization products (Sarkanen and Ludwig, 1971). Such precautions are not needed for cell wall studies because, as noted earlier, esterification of ferulate to xylans limits oxidative coupling reactions to the formation of dimers. In other studies, treatment of various FA esters with oxidases or one-electron oxidants invariably produces mostly 8−5-coupled products. Significant quantities of 5−5-coupled DFA are, however, formed only when FA is esterified to polysaccharides (Grabber et al., 1995; Ralph et al., 1992a, 1998; Wallace and Fry, 1995; Oosterveld et al., 1997; Schooneveld-Bergmans et al., 1999; Andreasen et al., 2000). The distribution of FA coupling products is controlled, in part, by the unpaired electron distribution in radicals and by the relative energies of the coupling products (Elder and Ede, 1995; Russell et al., 1996). In cell walls, the attachment and

Table 1. Fate of FA and DFA during Polymerization of Coniferyl Alcohol into Nonlignfied Maize Walls[a]

		FA	8−8-DFA	8−5-DFA	8-*O*−4-DFA	5−5-DFA
nonlignified walls	mg g^{-1}	7.5 ± 0.2	1.9 ± 0.2	6.3 ± 0.8	1.9 ± 0.2	2.1 ± 0.3
incorporated into lignin	mg g^{-1}	6.9 ± 0.2	1.5 ± 0.2	4.5 ± 0.8	1.6 ± 0.2	2.0 ± 0.3
	%	91.9[a]	78.8[bc]	71.5[c]	85.2[ab]	93.5[a]
ether linked to lignin	mg g^{-1}	2.4 ± 0.3	0.8 ± 0.1		1.0 ± 0.1	0.5 ± 0.2
	%	34.0[b]	56.2[a]		66.0[a]	26.4[b]

[a] Cell walls from two experiments were lignified to an average Klason lignin content of 138 mg g^{-1}. Due to modifications of some geometric and structural isomers during hydrolysis, only sums of isomers are presented. The release of ether-linked 8−5-coupled DFA could not be estimated. Percentages within the same row not followed by the same letter are significantly different according to LSD ($p = 0.05$).

the intra- and intermolecular distribution of FA on xylans probably alters the spatial interactions of FA radicals and the thermodynamics of bond formation and, as a result, the distribution of DFA coupling products. Such matrix effects probably account for the formation of 5−5-coupled DFA in cell walls. Indeed, molecular modeling studies by our group indicate that 5−5-coupled DFA may be the only DFA capable of being formed by intramolecular dimerization of FA polysaccharide esters (Hatfield and Ralph, 1999).

In total, 62% of wall ferulates were dimerized by hydrogen peroxide treatment (Table 1), which is somewhat higher than we observed in a previous study (Grabber et al., 1995). This high degree of dimerization is remarkable considering the low concentration of FA (~2%) in primary maize walls. Variations in cell wall feruloylation do not affect the extent of DFA formation (Grabber et al., 1995, 1998a). These observations suggest that FA are clustered or laid down at regular intervals within walls and in close proximity to peroxidases, permitting extensive DFA cross-linking of xylans at the onset of lignification or at other times when hydrogen peroxide is supplied to the apoplastic space. As discussed in the following section, xylans subsequently undergo additional cross-linking to lignin as FA monomers and dimers copolymerize with monolignols.

Although 40% of cell wall FA did not form DFA, most of these FA did subsequently undergo oxidative coupling with coniferyl alcohol during lignification. This suggests that FA accessibility and peroxidase availability are not major factors limiting DFA formation in primary maize walls. Rather, some FA are probably not in close enough proximity to one another to undergo oxidative coupling.

Incorporation of FA and DFA into lignin. Nonlignified maize walls were pretreated with an excess of H_2O_2 to complete DFA formation by wall-bound peroxidase and then slowly lignified with H_2O_2 and 9−143 mg g^{-1} of coniferyl alcohol (**13**). As in previous studies (Grabber et al., 1996), coniferyl alcohol was efficiently polymerized into wall-bound lignins; Klason lignin values exceeded the predicted lignin content of cell walls by ~15% (Figure 4). Klason lignin values are probably inflated by incorporation of ferulates, structural polysaccharides, and proteins to lignin (Grabber et al., 1996). Because oxidative coupling of FA and DFA esters with coniferyl alcohol prevents their release upon saponification, their incorporation into lignin can be estimated as the difference in alkali-labile acids recovered from nonlignified and lignified walls. The overall incorporation of FA monomers and dimers into lignin was rapid and extensive (Figure 4 and Table 2). Half of these FA (9.8 mg g^{-1}) were incorporated by the addition of only 20 mg g^{-1} of coniferyl alcohol, whereas an incorporation of 90% (17.6 mg g^{-1}) required the addition of nearly 200 mg g^{-1} of coniferyl alcohol. Most FA and DFA isomers began incorporation at the start of lignification, and the

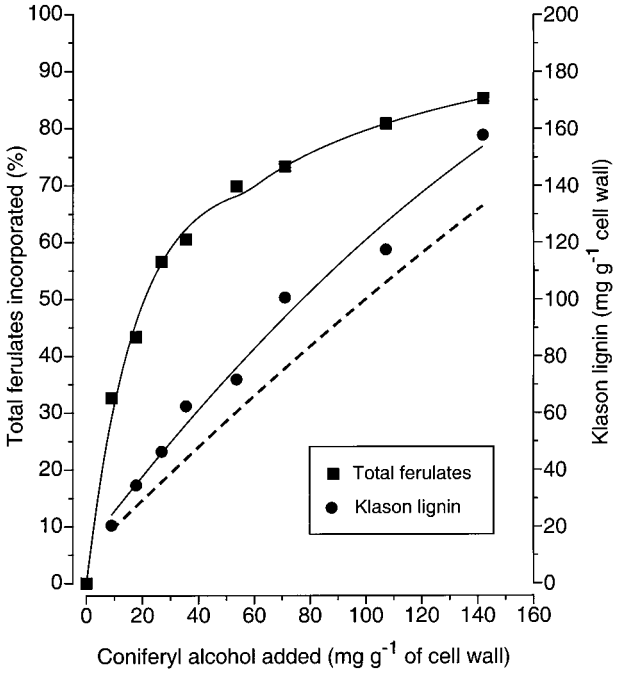

Figure 4. Overall incorporation of FA monomers and dimers (total ferulates) and changes in lignin content during lignification of primary maize walls with coniferyl alcohol. The dashed line indicates the predicted lignin content of cell walls.

final extent of incorporation generally exceeded 90% (Figures 5 and 6; Table 2). All isomers incorporated as two pools with a large pool (A1) incorporating earlier and more rapidly than a small pool (A2). Incorporation rates ranged from 0.022 to 0.144 for the A1 pool and from 0.011 to 0.040 for the A2 pool. Isomers with high A1 incorporation rates also tended to have high A2 incorporation rates. Rapidly incorporated isomers also tended to have larger A1 pools, smaller A2 pools, and an earlier incorporation of the A2 pool than slowly incorporated isomers. These pools may arise due to FA being located in regions of differing accessibility within cell walls.

A small amount of FA and DFA is probably inaccessible within cell walls; ~5% of FA in these walls cannot be methylated with diazomethane (Grabber et al., 1998b), and this corresponds to the amount of FA not incorporated into lignin (Table 2). The lignification process may further limit incorporation, especially of less reactive isomers, by inactivating peroxidases and by encrusting and dehydrating the cell wall matrix.

Among the structural isomers, 5−5-coupled DFA and FA monomers had the most rapid incorporation rates, reaching 90% incorporation into lignin with coniferyl alcohol additions of only 56 and 86 mg g^{-1}, respectively (Figure 5 and Table 2). In contrast, dehydrodimers coupled at the 8-position had a lower propensity to

Table 2. Kinetic Parameters Describing the Relationship between the Incorporation of FA and DFA into Lignin and the Quantity of Coniferyl Alcohol Polymerized into Cell Walls[a]

constituent	L_1	k_1	A1	L_2	k_2	A2	Fit
overall incorporation							
FA and DFA	0.0	0.058	71.3	57.2	0.011	23.7	0.995
incorporation of structural isomers							
FA	0.0	0.097	79.3	31.4	0.026	14.1	0.998
5−5-DFA	0.0	0.097	86.2	35.1	0.022	11.5	0.999
8−*O*−4-DFA	2.9	0.055	77.7	68.5	0.015	14.0	0.999
8−8-DFA	0.0	0.026	74.0	94.3	0.011	24.0	0.994
8−5-DFA	0.0	0.022	67.4	97.4	0.012	22.7	0.997
incorporation of geometric isomers							
(*E*)-FA	0.0	0.105	79.3	32.3	0.028	14.0	0.997
(*Z*)-FA	0.7	0.058	86.2	55.5	0.016	7.8	0.999
(*E*,*E*)-5−5-DFA	0.0	0.144	84.6	29.3	0.040	12.7	0.993
(*E*,*Z*)-5−5-DFA	0.0	0.083	72.3	26.8	0.013	27.1	0.999
(*E*)-8−5-DFA	0.0	0.031	61.4	95.9	0.031	17.7	0.996
(*Z*)-8−5-DFA	16.8	0.022	61.2	90.5	0.016	25.9	0.995

[a] Data were fitted to the dual-pool exponential model $y = A1[1 - e^{-k_1(CA-L_1-|L_1-CA|)}] + A2[1 - e^{-k_2(CA-L_2-|L_2-CA|)}]$, where CA = coniferyl alcohol added to cell walls (mg g^{-1}), A1 = rapidly incorporated pool (%), k_1 = rate of A1 incorporation, L_1 = CA added prior to A1 incorporation, A2 = slowly incorporated pool (%), k_2 = rate of A2 incorporation, and L_2 = CA added prior to A2 incorporation. Fit was calculated as 1 − (residual sum of squares/total degree-of-freedom corrected sum of squares).

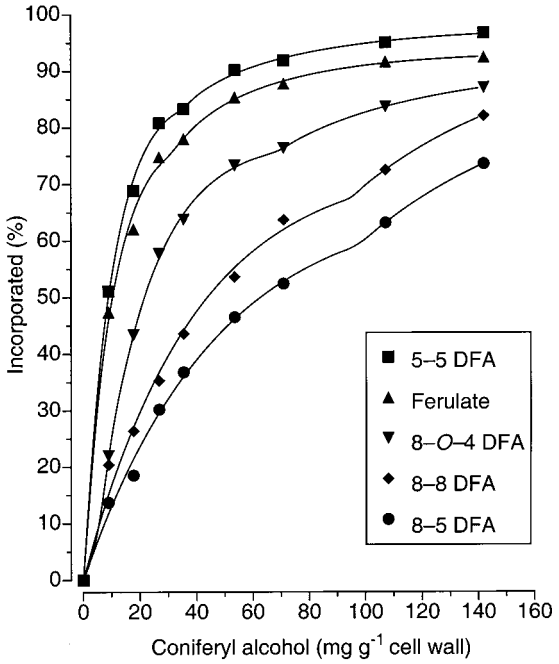

Figure 5. Incorporation of FA and DFA structural isomers during lignification of primary maize walls. FA were predimerized, prior to lignification, by incubating cell walls with dilute hydrogen peroxide.

copolymerize with coniferyl alcohol; 90% incorporation of 8−8-, 8−*O*-4-, and 8−5-coupled DFA required additions of 200, 210, and >300 mg g^{-1} of coniferyl alcohol, respectively. Geometric isomers in walls also differed in their rates of incorporation (Figure 6 and Table 2); (*E*)-FA **1b** was more rapidly incorporated into lignin than (*Z*)-FA **2b**, as were (*E*)- or (*E*,*E*)-DFA **7b** and **8b** than their corresponding (*Z*)- or (*E*,*Z*)-isomers **9b** and **11b**.

The propensity of FA and several DFA esters to copolymerize with coniferyl alcohol in vitro was similar to that observed under markedly different in situ conditions with cell wall esters (Table 3). In both

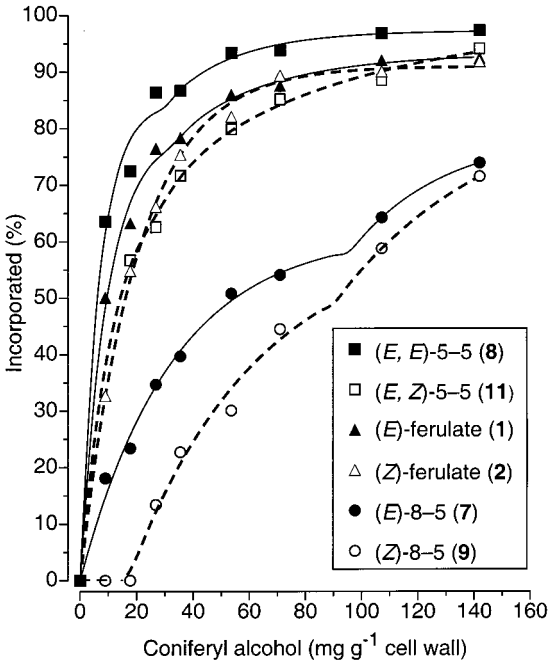

Figure 6. Incorporation of FA and DFA geometric isomers during lignification of primary maize walls. Ferulates were predimerized, prior to lignification, by incubating cell walls with dilute hydrogen peroxide.

Table 3. Percentage of Soluble Ethyl Esters and Wall-Bound Xylan Esters of FA and DFA Copolymerized with Coniferyl Alcohol at an Early Stage of Synthetic Lignin Formation[a]

	FA (**1**)	8−5-DFA (**7**)	5−5-DFA (**8**)
soluble ethyl esters	42.8[b]	17.4[c]	72.3[a]
insoluble xylan esters in walls	65.0[b]	28.1[c]	81.2[a]

[a] Ethyl esters were rapidly copolymerized with 20 equiv of coniferyl alcohol for 10 min using maize peroxidase and H_2O_2. Xylan esters in maize walls were slowly copolymerized with 1 equiv of coniferyl alcohol over a 60 min period using wall-bound peroxidase and H_2O_2 gradually generated by glucose oxidase. Means within the same row not followed by the same letter are significantly different according to LSD ($p = 0.05$).

systems, the rate of incorporation was most rapid for 5−5-coupled DFA, intermediate for FA, and lowest for 8−5-coupled DFA. On the basis of the work of Syrjanen and Brunow (1998, 2000), it appears that the esters incorporated in order from lowest to highest oxidation potential. These results suggest also that cell wall matrix effects do not greatly influence the initial rates of FA and DFA incorporation into lignin. Syrjanen and Brunow (1998, 2000) also suggest that cross-coupling occurs most readily between phenols of similar oxidation potential. Under the rapid polymerization conditions of the in vitro experiments, ethyl esters would encounter mostly dimers (and probably oligomers) of coniferyl alcohol (Sarkanen and Ludwig, 1971). In contrast, under the gradual polymerization conditions used for in situ wall studies, xylan esters would encounter mostly monomers of coniferyl alcohol (Sarkanen and Ludwig, 1971). Although coniferyl alcohol monomers and dimers differ in their oxidation potentials, this did not appear to alter the relative incorporation rates of FA and DFA in our in vitro and in situ experiments.

As a result of their differing incorporation rates, FA and DFA copolymerize with monolignols throughout the course of cell wall lignification, helping to anchor and

cross-link lignin to the polysaccharide matrix. Extensive xylan−xylan and xylan−lignin cross-linking by FA probably enhances wall strength (Iiyama et al., 1994) and reduces the susceptibility of walls to enzymatic hydrolysis (Grabber et al., 1998a,b).

Solvolytic Release of FA and DFA from lignin. High-temperature alkaline hydrolysis (4 M aqueous NaOH with anthraquinone at 170 °C) cleaves 4−*O*−*β*′ and 4−*O*−α′ ether-linked structures in lignin (Dimmel and Schuller, 1986a,b). FA and DFA involved in these structures (**14** and **15**) are also released (Iiyama et al., 1990; Lam et al., 1992), providing a means of estimating FA−lignin and DFA−lignin cross-linking in grasses. Unfortunately, FA and DFA coupled to lignin by 8−*O*-4′ styryl ethers or C−C bonds are not released by this or any other solvolytic method currently in use (Grabber et al., 1995). FA monomers and dimers are also degraded under these conditions, and such losses are difficult to estimate with any certainty. Therefore, these analyses underestimate, to an unknown degree, the extent of FA− and DFA−lignin cross-linking in lignified grass tissues. The abundance of these cross-links can, however, be estimated in our cell wall model system because the extent of FA and DFA incorporation is known and corrections can be made for the degradation of these acids during alkaline hydrolysis (see Materials and Methods).

High-temperature alkaline hydrolysis released ∼30% of the FA monomers and 5−5-coupled DFA incorporated into lignin (Table 1). The 8-coupled DFA had a greater propensity to form ether-linked structures with lignin; ∼60% of the 8−8- and 8−*O*-4-coupled DFA incorporated into lignin were released by high-temperature hydrolysis. The release of 8−5-coupled DFA from lignin could not be satisfactorily determined because a variable proportion of the decarboxylated product **12a** is derived from 8−5-coupled DFA and from a 5−*β*′-coupled cross-product of FA and coniferyl alcohol. Data for geometric isomers are not presented because high-temperature alkaline hydrolysis (unlike room temperature saponification) converts (*Z*) side chains of FA **2b** and DFA **9b** and **11b** to thermodynamically more stable acids with (*E*) configurations. These severe hydrolysis conditions also completely convert the 8−5-coupled benzofuran **7b** to structures **5a** and **12a** and most of the 8−8-coupled furan structure **10b** to structure **3a** or **4a.** As with saponification, 8−8-coupled DFA **3b** and **4b**, 8−*O*-4-coupled DFA **6b**, and 5−5-coupled DFA **8b** were recovered unmodified as acids following high-temperature alkaline hydrolysis.

Although mechanisms controlling radical coupling reactions of phenylpropanoids are only partially understood, FA incorporation into ether-linked structures may be low because FA couples at three sites (the 4−*O*-, 5-, and 8-positions). Studies with various phenylpropanoids indicate that coupling at the 4−*O*-position would not be favored (Sarkanen and Ludwig, 1971; Russell et al., 1996; Syrjanen and Brunow, 1998, 2000). In contrast, 8−*O*-4-coupled DFA (and probably 8−5-coupled DFA) may form a high proportion of ether-linked structures because coupling occurs at only two sites (the 4−*O*- and 5-positions) on one FA moiety. Studies with various mono- and dilignol models indicate that coupling at the 4−*O*-position would predominate (Sarkanen and Ludwig, 1971; Syrjanen and Brunow, 1998, 2000). Both 5−5- and 8−8-coupled DFA form cross-coupled structures at up to four sites (4−*O*- and the 8- or 5-positions of both FA moieties), potentially making these dimers more extensively cross-linked and less releasable after hydrolysis of ether linkages. Upon oxidation of one FA moiety, 5−5-coupled DFA readily forms α- and *β*-ether-linked dibenzodioxocin structures, preventing further incorporation of this dimer into lignin (Quideau and Ralph, 1997). Dibenzodioxocin structures, lacking 8-coupled units, should release 5−5-coupled DFA upon high-temperature hydrolysis (Karhunen et al., 1999). The low recovery of 5−5-coupled DFA after high-temperature hydrolysis suggests that this dimer is usually coupled to lignin via the 8-position of one or both of its side chains prior to the formation of any dibenzodioxocin structures.

In conclusion, primary walls in maize become extensively cross-linked by oxidative coupling of FA monomers into dehydrodimers followed by incorporation of FA monomers and dehydrodimers into lignin. FA monomers and dehydrodimers differ substantially in their propensity to copolymerize with coniferyl alcohol and to form hydrolyzable ether-linked structures with lignin. As a result, cross-links are formed throughout the course of lignification. Variable proportions of these cross-links are measurable by solvolytic techniques.

ACKNOWLEDGMENT

We thank Gundolf Wende, Jane Marita, and Mirko Bunzel for contributing mass spectra of diferulates and Dave Mertens for assistance with curve fitting. We are grateful to Rodger Peterson and David Snyder for running chemical ionization mass spectrometry and fast atom bombardment mass spectrometry of maize extracts.

LITERATURE CITED

Akin, D. E.; Hartley, R. D.; Rigsby, L. L.; Morrison III, W. H. Phenolic acids released from bermudagrass (*Cynodon dactylon*) by sequential sodium hydroxide treatment in relation to biodegradation of cell types. *J. Sci. Food Agric.* **1992**, *58*, 207−214.

Andreasen, M. F.; Christensen, L. P.; Meyer, A. S.; Hansen, A. Ferulic acid dehydrodimers in rye (*Secale cereale* L.). *J. Cereal Sci.* **2000**, *31*, 303−307.

Dimmel, D. R.; Schuller, L. F. Structural/reactivity studies. I. Soda reactions of lignin model compounds. *J. Wood Chem. Technol.* **1986a**, *6*, 535−564.

Dimmel, D. R.; Schuller, L. F. Structural/reactivity studies. II. Reactions of lignin model compounds with pulping additives. *J. Wood Chem. Technol.* **1986b**, *6*, 565−590.

Elder, T.; Ede, R. M. Coupling of coniferyl alcohol in the formation of dilignols: A molecular orbital study. *Proceedings of the 8th International Symposium of Wood and Pulping Chemistry*; Gummerus Kirjapaino Oy: Helsinki, Finland, 1995; Vol. 1, pp 115−122.

Gonzalez, L. F.; Rojas, M. C.; Perez, F. J. Diferulate and lignin formation is related to biochemical differences of wall-bound peroxidases. *Phytochemistry* **1999**, *50*, 711−717.

Grabber, J. H.; Hatfield, R. D.; Ralph, J.; Zon, J.; Amrhein, N. Ferulate cross-linking in cell walls isolated from maize cell suspensions. *Phytochemistry* **1995**, *40*, 1077−1082.

Grabber, J. H.; Ralph, J.; Hatfield, R. D.; Quideau, S.; Kuster, T.; Pell, A. N. Dehydrogenation Polymer-Cell Wall Complexes as a Model for Lignified Grass Walls. *J. Agric. Food Chem.* **1996**, *44*, 1453−1459.

Grabber, J. H.; Hatfield, R. D.; Ralph, J. Diferulate cross-links impede the enzymatic degradation of nonlignified maize walls. *J. Sci. Food Agric.* **1998a**, *77*, 193−200.

Grabber, J. H.; Ralph, J.; Hatfield, R. D. Ferulate cross-links limit the enzymatic degradation of synthetically lignified

primary walls of maize. *J. Agric. Food Chem.* **1998b**, *46*, 2609−2614.
Hartley, R. D.; Jones, E. C. Diferulic acid as a component of cell walls of *Lolium multiflorum*. *Phytochemistry* **1976**, *15*, 1157−1160.
Hartley, R. D.; Morrison, W. H., III; Balza, F.; Towers, G. H. N. Substituted truxillic and truxinic acids in cell walls of *Cynodon dactylon*. *Phytochemistry* **1990**, *29*, 3699−3703.
Hatfield, R. D.; Ralph, J. Modeling the feasibility of intramolecular dehydrodiferulate formation in grass walls. *J. Sci. Food Agric.* **1999**, *79*, 425−427.
Iiyama, K.; Lam, T. B. T.; Stone, B. A. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochemistry* **1990**, *29*, 733−737.
Iiyama, K.; Lam, T. B. T.; Stone, B. Covalent cross-links in the cell wall. *Plant Physiol.* **1994**, *104*, 315−320.
Ishii, T. Isolation and characterization of a diferuloyl arabinoxylan hexasaccharide from bamboo shoot cell-walls. *Carbohydr. Res.* **1991**, *219*, 15−22.
Jacquet, G.; Pollet, B.; Lapierre, C.; Mhamdi, F.; Rolando, C. New ether-linked ferulic acid-coniferyl alcohol dimers identified in grass straws. *J. Agric. Food Chem.* **1995**, *43*, 2746−2751.
Karhunen, P.; Mikkola, J.; Parjunen, A.; Brunow, G. The behaviour of dibenzodioxocin structures in lignin during alkaline pulping processes. *Nordic Pulp Paper Res. J.* **1999**, *14*, 123−128.
Lam, T. B. T.; Iiyama, K.; Stone, B. A. Changes in phenolic acids from internode walls of wheat and Phalaris during maturation. *Phytochemistry* **1992**, *31*, 2655−2658.
Markwalder, H. U.; Neukom, H. Diferulic acid as a possible cross-link in hemicelluloses from wheat germ. *Phytochemistry* **1976**, *15*, 836−837.
Musel, G.; Schindler, T.; Bergfeld, R.; Ruel, K.; Jacquet, G.; Lapierre, C.; Speth, V.; Schopfer, P. Structure and distribution of lignin in primary and secondary cell walls of maize coleoptiles analyzed by chemical and immunological probes. *Planta* **1997**, *201*, 146−159.
Myton, K. E.; Fry, S. C. Dithiothreitol and cobalt effects on membrane-associated peroxidases oxidizing feruloyl-CoA. *Phytochemistry* **1995**, *38*, 573−577.
Oosterveld, A.; Grabber, J. H.; Beldman, G.; Ralph, J.; Voragen, A. G. J. Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin. *Carbohydr. Res.* **1997**, *300*, 179−181.
Quideau, S.; Ralph, J. Lignin-ferulate cross-links in grasses. Part 4. Incorporation of 5−5-coupled diferulate into lignin. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2351−2358.
Ralph, J.; Zhang, Y. A new synthesis of *(Z)*-coniferyl alcohol, and characterization of its derived synthetic lignin. *Tetrahedron* **1998**, *54*, 1349−1354.
Ralph, J.; Helm, R. F.; Quideau, S. Lignin-feruloyl ester cross-links in grasses. Part 2. Model compound syntheses. *J. Chem. Soc., Perkin Trans. 1* **1992a**, 2971−2980.
Ralph, J.; Helm, R. F.; Quideau, S.; Hatfield, R. D. Lignin-feruloyl ester cross-links in grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. *J. Chem. Soc., Perkin Trans. 1* **1992b**, 2961−2969.
Ralph, J.; Quideau, S.; Grabber, J. H.; Hatfield, R. D. Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. *J. Chem. Soc., Perkin Trans. 1* **1994**, 3485−3498.
Ralph, J.; Grabber, J. H.; Hatfield, R. D. Lignin-ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydr. Res.* **1995**, *275*, 167−178.
Ralph, J.; Garcia-Conesa, M. T.; Williamson, G. Simple preparation of 8−5-coupled diferulate. *J. Agric. Food Chem.* **1998**, *46*, 2531−2532.
Russell, W. R.; Forrester, A. R.; Chesson, A.; Burkitt, M. J. Oxidative coupling during lignin polymerization is determined by unpaired electron delocalization within parent phenylpropanoid radicals. *Arch. Biochem. Biophys.* **1996**, *332*, 357−366.
Sarkanen, K. V.; Ludwig, C. H. *Lignins, Occurrence, Formation, Structure and Reactions*; Wiley-Interscience: New York, 1971.
Schooneveld-Bergmans, M. E. F.; Dignum, M. J. W.; Grabber, J. H.; Beldman, G.; Voragen, A. G. J. Studies on the oxidative cross-linking of feruloylated arabinoxylans from wheat flour and wheat bran. *Carbohydr. Polym.* **1999**, *38*, 309−317.
Schopfer, P. Hydrogen peroxide-mediated cell-wall stiffening in vitro in maize coleoptiles. *Planta* **1996**, *199*, 43−49.
Syrjanen, K.; Brunow, G. Oxidative cross coupling of *p*-hydroxycinnamic alcohols with dimeric arylglycerol *β*-aryl ether lignin model compounds. The effect of oxidation potentials. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3425−3429.
Syrjanen, K.; Brunow, G. Regioselectivity in lignin biosynthesis. The influence of dimerization and cross-coupling. *J. Chem. Soc., Perkin Trans. 1* **2000**, 183−187.
Tan, K.-S.; Hoson, T.; Masuda, Y.; Kamisaka, S. Involvement of cell wall-bound diferulic acid in light-induced decrease in growth rate and cell wall extensibility of *Oryza* coleoptiles. *Plant Cell Physiol.* **1992**, *33*, 103−108.
Turner, L. B.; Mueller-Harvey, I.; McAllan, A. B. Light-induced isomerization and dimerization of cinnamic acid derivatives in cell walls. *Phytochemistry* **1993**, *33*, 791−796.
Wallace, G.; Fry, S. C. In vitro peroxidase-catalyzed oxidation of ferulic acid esters. *Phytochemistry* **1995**, *39*, 1293−1299.

Received for review June 6, 2000. Revised manuscript received October 16, 2000. Accepted October 17, 2000. Supported in part by USDA-NRI Competitive Grants 94-37500-0580 (Enhancing Value and Use of Agricultural and Forest Products) and 96-35304-3864 (Plant Growth and Development).

JF0006978